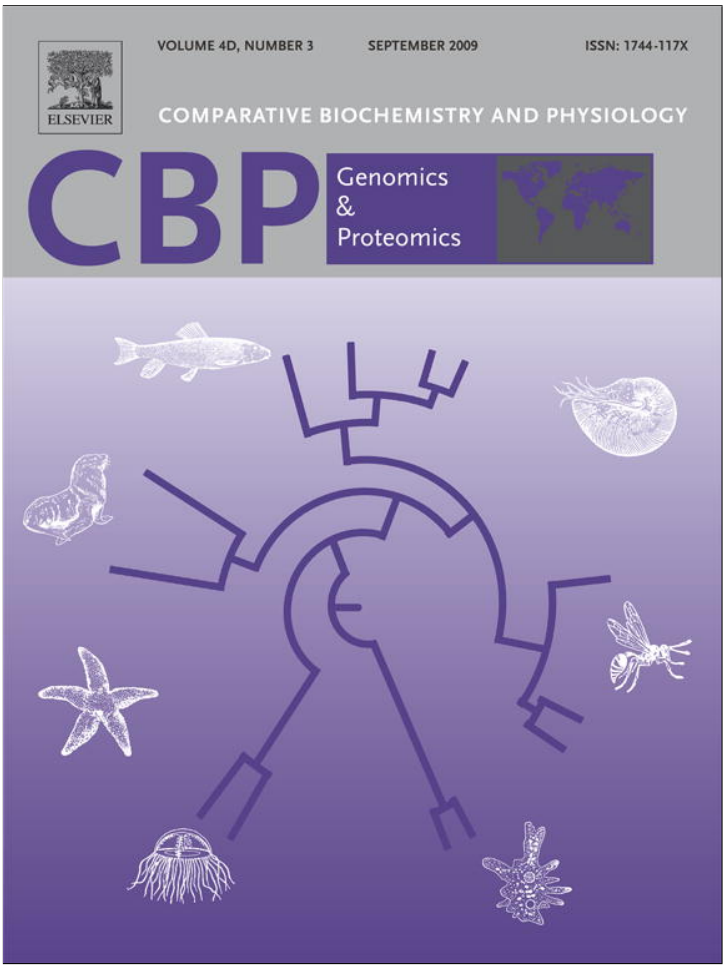

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Transcriptomic profiling of *Ichthyophthirius multifiliis* reveals polyadenylation of the large subunit ribosomal RNA

Jason W. Abernathy [a], De-Hai Xu [b], Ping Li [a], Phillip Klesius [b], Huseyin Kucuktas [a], Zhanjiang Liu [a,*]

[a] The Fish Molecular Genetics and Biotechnology Laboratory, Department of Fisheries and Allied Aquacultures and Program of Cell and Molecular Biosciences, Aquatic Genomics Unit, 203 Swingle Hall, Auburn University, AL 36849, USA
[b] Aquatic Animal Health Research Laboratory, Agricultural Research Service, United States Department of Agriculture, 990 Wire Road, Auburn, AL 36831, USA

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ABSTRACT

Polyadenylation of eukaryotic transcripts is usually restricted to mRNA, providing transcripts with stability from degradation by nucleases. Conversely, an RNA degradation pathway can be signaled through poly (A) tailing in prokaryotic, archeal, and organellar biology. Recently polyadenylated transcripts have also been discovered in rRNA in some eukaryotes including humans and yeast. Here we report the discovery of polyadenylated rRNAs in the ciliate teleost parasite *Ichthyophthirius multifiliis*, an important fish pathogen. Through large-scale analysis of ESTs, a large contig composed of the 28S rRNA with poly (A) tails was identified. Analysis using multiple sequence alignments revealed four potential polyadenylation sites including three internal regions and the 3′ end of the rRNA. Further analysis using a polyadenylation test, re-sequencing, and gene-specific PCR using primers flanking the presumed poly (A) sites confirmed the presence of polyadenylated rRNA in this parasite. The functions of polyadenylation of rRNA in this organism are largely unknown at present, but the presence of internal polyadenylation sites, along with the presence of truncated segments of the rRNA, may suggest a role of the polyadenylation in the degradation pathway, a function typical of prokaryotes, archaea, and organelles. These results are in congruence with reports of a similar phenomenon in humans and yeast.

1. Introduction

Three main classes of RNA exist in eukaryotes consisting of ribosomal RNA (rRNA), messenger RNA (mRNA), and transfer RNA (tRNA). In protein synthesis, rRNA and ribosomal proteins form the ribosomes where protein synthesis occurs; mRNAs encode the primary sequence of proteins for which the proper amino acids are transferred to the growing peptide chain by tRNA. In addition to these three major classes of RNAs, several other classes of RNAs have been reported including hybrid transfer-messenger RNA (tmRNA), small nuclear and small nucleolar RNA (snRNA and snoRNA), and micro-RNA (miRNA).

Important features of RNA are the modifications that occur during or immediately after transcription; in particular 5′ methylated reverse capping and 3′ polyadenylation. Polyadenylation is evident not only in the mRNA of all domains of eukaryotes but also in some organellar RNA: mitochondrial RNA (mtRNA) or chloroplast RNA (cpRNA). Typically, eukaryotic mRNA is polyadenylated on the mature transcript with implications for stability, signaling, and nuclear transport in compartmentalized cells, and can also serve as a binding domain (Beelman and Parker, 1995; de Moor and Richter, 2001; Edmonds, 2002; Mangus et al., 2003; Shatkin and Manley, 2000). In contrast, polyadenylation of mRNA in prokaryotes and archaea, as well as polyadenylation with certain mtRNA or cpRNA, signals RNA degradation (Dreyfus and Regnier, 2002, Kushner, 2004, O'Hara et al., 1995, Slomovic et al., 2005). In recent RNA examinations, exceptions to the stability versus decay rule are rising and coexisting mechanisms seem to occur (Kao and Read, 2005; LaCava et al., 2005; Slomovic et al., 2005; Vanacova et al., 2005; West et al., 2006; Wyers et al., 2005).

Polyadenylation is typically thought to be exclusive to translated RNA. Conversely, non-translated rRNAs have recently been shown to exhibit polyadenylation in some eukaryotes. Discoveries have been forthcoming of rRNA polyadenylation in humans (Slomovic et al., 2006), yeasts *Candida albicans* and *Saccharomyces cerevisiae* (Fleischmann and Liu, 2001; Fleischmann et al., 2004; Kuai et al., 2004), and the protozoan Kinetoplastid *Leishmania* (Decuypere et al., 2005). Here we report the polyadenylation of the large subunit rRNA in the ciliate protozoan *Ichthyophthirius multifiliis* (Ich).

The ciliate protozoan *I. multifiliis* is a devastating freshwater teleost parasite. The parasite is widespread; it affects many freshwater fish species around the world. It causes great loss to both the aquaculture and ornamental fish industries. Ich is responsible for the disease

* Corresponding author. Aquatic Genomics Unit, Department of Fisheries and Allied Aquacultures, 203 Swingle Hall, Auburn University, AL 36849, USA. Tel.: +1 334 844 4054; fax: +1 334 844 4694.
E-mail address: zliu@acesag.auburn.edu (Z. Liu).

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ichthyophthiriosis or 'white spot' disease with characteristic white spot cysts forming under the fish gill or epidermis. Also, typical of unicellular members of the phylum Ciliophora, Ich exhibits nuclear dimorphism. It possesses the characteristic macronucleus and micronucleus. Ich has been shown to be closely related phylogenetically to species of *Tetrahymena* (Abernathy et al., 2007; Eisen et al., 2006; Wright and Lynn, 1995) such as *T. thermophila*, a free-living non-parasitic ciliate protozoan. From a comparative view to *Tetrahymena* (Eisen et al., 2006; Prescott, 1994), Ich may also undergo both germ line and somatic cell divisions, generally where the micronucleus contains the germ line DNA that can be replenished through meiotic conjugation, and the macronuclear DNA that is transcriptionally active to support the cell. There has been evidence for asexual reproduction in Ich for many years, with possible evidence that some sexual regeneration may occur [for a review see Matthews, 2005]. One such piece of evidence arises from the major difficulties encountered in maintaining viable isolates of Ich in the laboratory setting. Multiple passages of an Ich isolate on a fish host lead to a significant decline in infectivity, relating to senescence (Matthews, 2005; Xu and Klesius, 2004). It is speculated that this type of induced senescence could be due to a lack of recombination of the germ line. Therefore, undertaking a study of senescence-related genes would be an important step in better understanding Ich development, reproduction, and parasitic nature.

A set of highly abundant transcripts containing poly (A) tails at the 3′ end was initially identified during EST analysis (Abernathy et al., 2007). BLASTX similarity comparisons of these transcripts yielded hypothetical proteins related to senescence proteins in antisense orientation, with low to moderate similarity ($e^{-6} \geq$ E-value $\geq e^{-78}$ for various segmented alignments). We continued to study these transcripts to test for senescence-related expression, and we learned that the antisense transcripts did not exist using Northern blot analysis, but its related sense strand transcripts were highly expressed in all three stages and all ages of Ich tested. Such results led to more extensive BLAST searches using both BLASTX and BLASTN. BLASTN searches revealed that these transcripts were highly similar (E-value = 0) to ribosomal RNAs, in particular to portions of the large subunit ribosomal RNA in many organisms, including closely related *Tetrahymena* species, or to precursor-rRNA in *T. thermophila* with alignment to the large subunit. However, the putative identity as rRNA is in contradiction of the fact that the transcripts were found to be polyadenylated. Therefore, we set out to further characterize these transcripts, obtain more of their sequences, and test for polyadenylation. Here we present the 28S rRNA sequence from the ciliate protozoan *I. multifiliis*, and provide evidence for polyadenylation of the large subunit rRNA.

2. Materials and methods

2.1. Cells, RNA isolation, and analysis

A single Ich isolate was obtained from a local pet shop with an outbreak of ichthyophthiriosis. Ich was cultured on fish as previously described (Abernathy et al., 2007). Samples were washed in phosphate-buffered saline (PBS; pH 7.2) and flash-frozen in liquid nitrogen. Cells were stored in a −80 °C freezer until usage. Total RNA was isolated from each of the three Ich life-stages using the RNeasy Plus Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The kit uses a spin column to eliminate DNA contamination. RNA quality was assessed by using denaturing agarose gel electrophoresis containing formaldehyde (Liu, 2007a).

2.2. Sequence analysis

The Ich ESTs used for assembly of the initial contiguous sequences (contigs) are from GenBank dbEST accession numbers EG957858–EG966289. The ESTs were generated by sequencing a unidirectional cDNA library enriched for full-length cDNAs (Abernathy et al., 2007; Liu, 2007a,b). All ESTs were sequenced from the 5′ end. The sequences were assembled into contigs using ContigExpress in the Vector NTI version 10.3.0 software (Invitrogen, Carlsbad, CA, USA) with an overlap length cutoff set at 40 bp and sequence identity cutoff of the overlap set at 90%. All other settings were at the default values. The contig used for this study was the largest contig in the assembly containing 764 ESTs. The contig was visually inspected for polyadenylated regions. During the course of this study, EST resources grew significantly in the dbEST database of the GenBank. Using the latest release, version 031408 (25,084 ESTs), we were able to assemble the contig to include the mature 3′ end of the rRNA molecule as compared with the top 4 megablast hits.

Sequence identity was determined using BLASTN at the National Center for Biotechnology Information (NCBI). Multiple sequence alignments were conducted using ClustalW with the 2677 bp Ich consensus sequence from the contig (herein 28S rRNA), along with the top homologous sequences generated from BLAST searches.

2.3. Northern blot

Two separate Northern blot analyses (Sambrook et al., 1989) were performed on the three life-stages of Ich. Briefly, total RNA was isolated using the RNeasy Plus kit according to the manufacturer's provided protocol. Total RNA (3 μg each of the three Ich life-stages) was separated by electrophoreses on a 1% agarose gel containing formaldehyde. The gel was UV visualized, then rinsed twice for 10 min in DEPC-treated water to destain and remove excess formaldehyde. The gel was dipped in 20× SSC buffer. A downward capillary transfer was performed for 4 h using positively charged nylon membranes (Millipore, Bedford, MA) and 20× SSC buffer. After transfer, the membranes were dipped in 20× SSC buffer, and RNA was fixed to the membrane by UV-crosslink using the UV Stratalinker 2400 (Stratagene, La Jolla, CA, USA) using the auto-crosslink setting. The membranes were pre-hybridized in 5 mL ULTRAhyb-Oligo buffer (Ambion, Austin, TX, USA) for 1.5 h at 42 °C. Labeled probes were added and hybridized overnight at 42 °C in a hybridization oven. The membranes were washed twice for 30 min each in a wash buffer (2× SSC, 0.5% SDS) and exposed to X-ray film for 4 h at room temperature. As our initial BLASTX analysis indicated the contig under consideration was similar to senescence proteins, but in an antisense orientation, two probes were used to determine the presence of sense-strand transcript (using an antisense probe: 5′-GACCAGAGGCTGCTAACCTTGGAGACCTGATGCGGTTATG-3′) and antisense transcript (using a sense probe: 5′-CATAACCGCATCAGGTCTCCAAGGTTAGCAGCCTCTGGTC-3′). The probes were end-labeled using ATP [γ-^{32}P] (MP Biomedicals, Solon, OH, USA).

2.4. Testing for polyadenylation at the full 3′ end

One-hundred nanograms of total RNA from each of the three Ich life-stages was pooled and reverse transcribed using an oligo (dT) adapter primer 5′-GGTGAGCCCGCGTCACGG$(T)_{12}$-3′ as designed elsewhere (Decuypere et al., 2005) using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's protocol. A polyadenylation test was conducted as previously described (Salles

Table 1
PCR primer sequences and product sizes assuming there are no internal poly (A/T) tracts as predicted from the consensus sequences.

	Upper primer	Lower primer	Predicted PCR product sizes	Observed PCR product sizes
First poly (A) site	GGTCTCCAAGGTTAGCAGC	AGGCCGAAGCCACTCTAC	200 bp	200 bp
Second poly (A) site	ACATGCCTGCGCATAAG	GTTGAATTGCGTCACTTTGA	200 bp	200 bp
Third poly (A) site	TGCCGTGAAGCTACCATC	GACTCTTTCGTCTTCAGCC	150 bp	150 bp

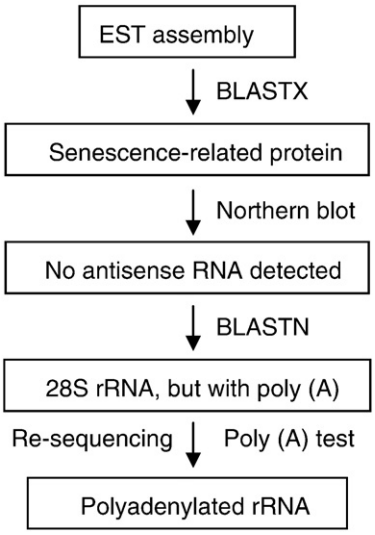

Fig. 1. Flowchart of molecular analysis leading to the identification of the polyadenylated 28S rRNA.

Table 2
The top 10 megablast BLASTN hits to the Ich transcript.

Accession number	Description	Identity	E-value
X54004	*Tetrahymena pyriformis* gene for 26S large subunit ribosomal RNA	92%	0
AY210458	*Crisia* sp. YJP-2003 28S ribosomal RNA gene, partial sequence	84%	0
X54512	*T. thermophila* rdn A+ gene for pre-RNA, 17S rRNA, 5.8S rRNA, 26S rRNA	91%	0
DQ273766	*Rozella* sp. JEL347 isolate AFTOL-ID 16 28S ribosomal RNA gene, partial sequence	82%	0
DQ273770	*Rhizophydium brooksianum* isolate AFTOL-ID 22 28S ribosomal RNA gene, partial sequence	81%	0
DQ273825	*Rhizophydium* sp. PL 42 isolate AFTOL-ID 691 28S ribosomal RNA gene, partial sequence	81%	0
DQ273823	*R. macroporosum* isolate AFTOL-ID 689 28S ribosomal RNA gene, partial sequence	81%	0
AF149979	*Paramecium tetraurelia* macronuclear X gene, complete sequence	87%	0
XM_001471443	*T. thermophila* SB210 hypothetical protein (TTHERM_02141639) mRNA, complete cds	91%	0
DQ273835	*Rhizophydium* sp. JEL316 isolate AFTOL-ID 1535 28S ribosomal RNA gene, partial sequence	86%	0

et al., 1999). Briefly, RNA is reverse transcribed using an oligo (dT) adapter primer. This reaction was used as a template for the polyadenylation test. To test for polyadenylation, a PCR reaction was performed where the oligo (dT) adapter primer and a gene-specific primer designed close to the polyadenylated site, were used to amplify the gene. The oligo (dT) adapter primer had the ability to hybridize at any position along the poly (A) tail. In this regard, the polyadenylation test allowed the determination of the presence/absence as well as size differentiation of the poly (A) tail (Salles et al., 1999). The amplicon is subsequently digested by a restriction enzyme with a unique restriction site close to the 5′ end and analyzed on an agarose gel. For our analyses, a 6-bp cutter (*Acu* I, New England Biolabs, Ipswich, MA) was chosen as a restriction enzyme for its uniqueness and position in the amplicon. Restriction enzyme digestion reactions were conducted under standard conditions according to the manufacturer's instructions.

For Ich, the polyadenylation test was used to test polyadenylation at the full 3′ end of the 28S rRNA. We designed the polyadenylation test primer located at nucleotide position 2482–2502 of the consensus sequence. At this position, the test would help to determine if the added 3′ end was truly part of the gene at the 3′ end after clustering, and test for polyadenylated transcripts. One-hundred nanograms of total RNA from each of the three Ich life-stages was pooled and reverse transcribed using the oligo (dT) adapter primer. This first-strand reaction was used as a template for the polyadenylation test. A PCR reaction was performed using reaction guidelines from the polyadenylation test (Salles et al., 1999) with a gene-specific forward primer (GSP1, 5′-TAAGCGCAAGCTTAAGTTCGA-3′) and the oligo (dT) adapter primer as a reverse primer. The thermo profiles for the PCR reaction were: an initial heat treatment at 94 °C for 3 min, 94 °C for 30 s, 55 °C for 30 s, 72 °C for 3 min, for 35 cycles, followed by a final 7 min at 72 °C. Additional PCR cycles were added from the polyadenylation test in increments of 5 where needed to achieve an amplicon. The expected amplicon size was of a minimum size of 208 bp. The resulting product was digested with *Acu* I to produce a single band representing the 5′ end of the amplicon (expected size of 58 bp) and a 3′ polyadenylated end of at least 150 bp. The digested and undigested products were electrophoresed on a 2% agarose gel for visualization.

2.5. Testing for polyadenylation at internal sites

To test the internal sites of the 28S for polyadenylation, we chose to re-sequence and re-analyze some of the original clones that contained putative poly (A) tails, and that clustered internally of the 28S. A total of 10 clones from our cDNA library (Abernathy et al., 2007) were randomly selected for re-sequencing to test whether these clones truly contained poly (A) tails, and to test for sequencing artifacts from the original trace files. All clones selected were isolated from single bacterial colonies from −80 °C stocks, plasmid DNA obtained using the Qiaprep Spin Miniprep kit (Qiagen), and sequenced in triplicate using M13 (−21) universal primer and BigDye v3.1 on an ABI 3130*XL* DNA analyzer according to the manufacturer's recommendation (Applied Biosystems, Foster City, CA). To give the highest confidence that our sequences contain poly (A) tails, post-sequencing processing included base-calling using Phred (Ewing and Green, 1998; Ewing et al., 1998) statistics at >Q20 (>99% accuracy). Vector sequences were eliminated by VecScreen BLAST. The consensus of the triplicate sequence was used for each clone. These sequences were re-analyzed by clustering against the consensus 28S and using BLAST tools.

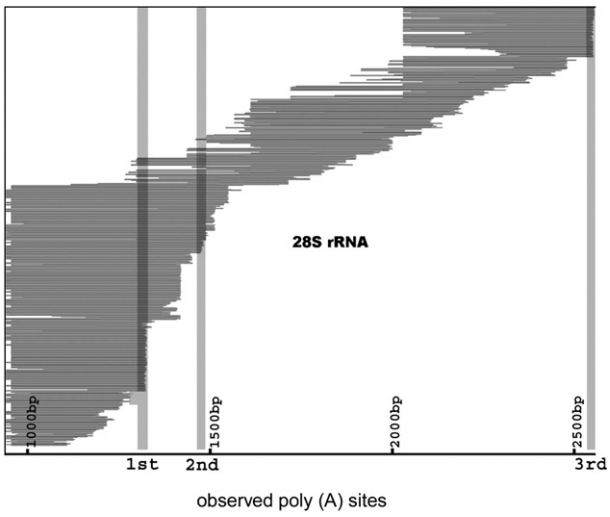

Fig. 2. Schematic presentation of the internal polyadenylation sites. Initial contig was assembled using Ich ESTs generated from GenBank dbEST sequences from EG957858–EG966289. Vertical shaded regions labeled as 1st, 2nd, and 3rd on the *X*-axis represent regions of observed internal polyadenylation of ESTs. Nucleotide positions are given based on the complete rRNA consensus sequence assembled with ContigExpress (Vector NTI 10.3.0 software, Invitrogen) with poly (A) tails included. Poly (A) sites were not detected within the first 1000 bp of the rRNA sequences, and therefore the schematic presentation is truncated for better exhibition of the detected poly (A) sites.

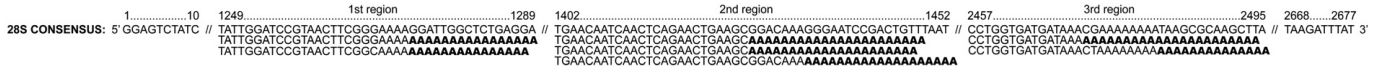

Fig. 3. Graphical results from re-sequencing clones at internal polyadenylated sites. The top sequence is the 28S rDNA consensus sequence (GenBank accession no. EU185635). Numbers along the top represent the base-pair position along the consensus 28S sequence. A total of 7 out of 10 clones that were re-sequenced contained a poly (A) tail after base-calling at >99% accuracy using phred >Q20. The 1st, 2nd, and 3rd polyadenylation regions are each represented. Double bars indicate areas where the sequence was shortened for brevity.

In addition to the direct sequencing analysis and informatic analysis, we also conducted PCR analysis of the region containing the presumed poly (A) sites using flanking PCR primers, with one PCR primer upstream and the other downstream of the presumed poly (A) sites. The rationale is that if poly (A/T) sequences do exist internally, then the size of the PCR products would be larger than the predicted size without internal poly (A/T) sequences based on our consensus sequences. PCR primer pairs were designed for the three presumed poly (A) sites, and PCR reactions were conducted with thermo cycling profiles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min with a total of 35 cycles. An initial 3 min of heat treatment at 94°C was used. The PCR products were analyzed on both 1.8% agarose gels and on 8% polyacrylamide gels (Sambrook et al., 1989). PCR primers used for the test are shown in Table 1.

3. Results

3.1. Identification of the largest contig in the EST assembly as the 28S rRNA

The overall strategy for the demonstration of the presence of polyadenylated 28S rRNA in *I. multifiliis* is shown in Fig. 1. As introduced above, initial BLASTX analysis generated several significant hits to existing proteins named senescence-related proteins, but in an antisense orientation. However, Northern blot analysis revealed that there was no antisense RNA present. Further BLAST analysis was conducted using both BLASTX and BLASTN. The BLASTN searches revealed that the identity of the contig under study was the large subunit of rRNA (Table 2). All E-values for the top 100 megablast hits were zero and identities were ≥81%. As expected, the 26S rRNA of the closely related ciliate *Tetrahymena pyriformis* and *T. thermophila* had the highest similarity, with ≥91% identity covering the entire rRNA transcripts. Sequence alignments further confirm the identity of the involved transcripts. Obviously, in the initial BLASTX searches, rRNA was not included in the protein database. However, this result also indicated the polyadenylation of the 28S rRNA transcripts in Ich, an unusual but reported phenomenon in several organisms such as yeast and humans (Fleischmann and Liu, 2001; Fleischmann et al., 2004; Kuai et al., 2004; Slomovic et al., 2006). A consensus sequence of the complete Ich 28S rRNA was generated by multiple sequence alignment using all ESTs within this contig, and the consensus sequence has been submitted to GenBank (accession number EU185635).

3.2. Polyadenylation of the Ich large subunit rRNA

The initial contig involving the transcript under study was assembled using ESTs we previously generated (Abernathy et al., 2007) and is depicted in Fig. 2. A total of 207 (27%) polyadenylated ESTs were found in this contig of 764 ESTs (note that all these 764 ESTs could contain poly (A) tails, but because the ESTs were sequenced from the 5′ end, many clones were not sequenced through the whole inserts). The regions containing the poly (A) tails fall into three regions, 1220–1279 bp, 1427–1450 bp, and 2468–2501 bp along the consensus sequence (Fig. 2). Further searches of GenBank (now containing over 33,000 EST sequences) allowed the identification of additional polyadenylated sequences that fall under these three regions, as well as polyadenylated sequences containing poly (A) tails at the real 3′ end of the rRNA transcripts. These results suggested the presence of polyadenylated 28S rRNA with poly (A) tails both at the 3′ end of the transcript and at three internal sites of the transcript.

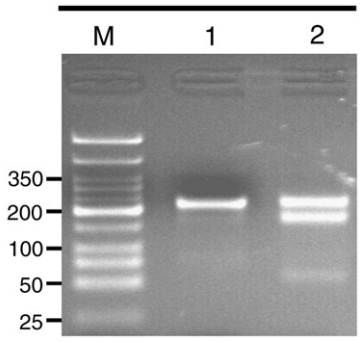

Fig. 4. Results of the polyadenylation test for polyadenylation at the 3′ end of the rRNA. Lane 1, undigested amplicon of the polyadenylation test; lane 2, the amplicon digested with restriction endonuclease *Acu* I, note that not all the amplified products were digested completely; and M, low molecular weight marker from New England Biolabs (NEB #N3233).

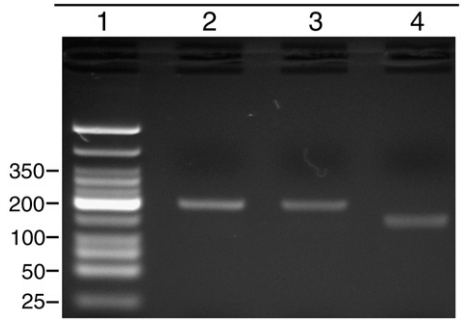

Fig. 5. PCR test for the presence of polyadenylation using gene-specific primers flanking each of the three internal poly (A) sites. Lane 1, low molecular weight (in bp) marker from NEB; lane 2–4, PCR products from the first, second, and third internal poly (A) sites, respectively.

Of the 207 observed clones with poly (A) tails, 113 clones had poly (A) at the first site; 8 clones had poly (A) at the second site; and 86 clones had poly (A) at the third site (Fig. 2). The majority of poly (A) tails had lengths of 9–23 bp after subtraction of the oligo (dT) primer of 12 bases. Sequence alignments of all the 764 clones clearly suggest that the poly (A) was not part of the gene internal sequences because we had many clones that were sequenced through the poly (A) junction, but there were no poly (A/T) being observed. Furthermore, the consensus sequence [without poly (A)] aligned nicely with the large subunit ribosomal RNA from closely related species (not shown). All these direct sequence data provide very strong evidence that polyadenylation was real rather than being derived from artifacts due to internal priming of the oligo (dT) primers during cDNA synthesis.

We also re-sequenced some of the original clones to test if the insert contained a poly (A) tail at the internal sites. Out of the 10 clones chosen for re-sequencing, 7 were found to contain poly (A) tails after stringent post-sequencing processing. All 3 internal sites were represented by these 7 clones. BLASTN was performed on each sequence, and all were determined to be a portion of the 28S rRNA gene. These sequences were then re-clustered against the consensus 28S sequence, and it was clear that these transcripts align to the 28S. As with the original 764 EST cluster described above, these sequences produced an alignment file with a consensus sequence containing transcripts with poly (A) overhangs (Fig. 3).

In addition to direct sequence alignments, additional tests were conducted to demonstrate the presence of polyadenylation in 28S rRNA transcripts. First, a polyadenylation test (Salles et al., 1999) was used to determine if the rRNA was truly 3′ polyadenylated, or the sequenced ESTs were just an artifact. PCR amplification was conducted using one rRNA gene-specific primer and one poly (T) primer, with the gene-specific primer designed proximal to the suspected poly (A) sites. The 3′ end of the 28S, determined after clustering and alignments with other similar species, was tested for potential polyadenylation with the polyadenylation test at the consensus sequence position 2677 bp (the full 3′ end). As shown in Fig. 4, the PCR product was amplified and correctly digested. The PCR product had the correct sizes as predicted from the potential poly (A) site,

Fig. 6. Sequence alignment of the 180-bp regions immediately upstream to each of the observed polyadenylation sites using ClustalW. The 1st, 2nd, and 3rd regions correspond to 1st, 2nd, and 3rd regions of polyadenylation sites in Fig. 3, whereas 3′ end indicates sequences immediately upstream from the 3′ end of the rRNA. Black highlights are conserved nucleotides and grey highlights are similar nucleotides. No shading indicates non-similarity.

confirming the correctness of the consensus sequence and the presence of polyadenylated transcripts within the pool of the 28S rRNA transcripts.

The poly (A) test is widely used in the literature for the validation of the presence of polyadenylation at the 3′ end of the transcripts (Salles et al., 1999; Fleischmann and Liu, 2001; Fleischmann et al., 2004; Kuai et al., 2004; Slomovic et al., 2006). However, for internal polyadenylation sites, one can argue that the PCR is supported by internal priming of the oligo (dT) primers. Therefore, another set of PCR tests was conducted to demonstrate that the observed poly (A) tails were not derived from internal priming of the oligo (dT) primers during cDNA synthesis. In this experiment, PCR primers were designed flanking the three presumed poly (A) sites such that the amplified segments would contain the poly (A) region. If the 28S rRNA gene contained internal poly (A/T) sequences, the PCR products would be longer than the consensus sequence. As shown in Fig. 5 and Table 1, the PCR products were exactly the same size as predicted from the consensus sequence without internal poly (A/T) sequences, providing strong supporting evidence that the poly (A)-containing clones from the EST analysis were not derived from internal priming.

4. Discussion

Polyadenylation typically occurs with mRNA of eukaryotic transcripts. Recent studies, however, have discovered polyadenylation in non-translated ribosomal RNA in a few organisms (Decuypere et al., 2005; Fleischmann and Liu, 2001; Fleischmann et al., 2004; Kuai et al., 2004; Slomovic et al., 2006). In humans and yeast, polyadenylated rRNA was reported for both 18S and 28S rRNAs (Fleischmann and Liu, 2001; Fleischmann et al., 2004; Kuai et al., 2004; Slomovic et al., 2006). In the protozoan parasite *Leishmania*, polyadenylation was found at several positions within the large subunit rRNA (Decuypere et al., 2005). Here we report the polyadenylation of the large subunit rRNA in the ciliate protozoan parasite *I. multifiliis*, at both the 3′ end and three internal positions within the 28S rRNA. To our best knowledge, this is the first report of this phenomenon in ciliate parasites.

The strongest supporting evidence for the presence of polyadenylated 28S rRNA comes from multiple sequence alignment where a consensus sequence can be assembled based on several hundreds of sequences, and among them, over 270 sequences were found to harbor poly (A) tails. The presence of EST sequences of clones with poly (A) at the 3′ end, and also clones spanning through the three internal poly (A) sites but without any poly (A) tract clearly demonstrates a polyadenylation in the internal sites. In order to confirm the polyadenylation of the 28S rRNA at the 3′ end and internally, a polyadenylation test was conducted using PCR as well as re-sequencing. The positive result of the polyadenylation test PCR provided further evidence for polyadenylation of the 3′ end of the 28S rRNA in Ich. Re-sequencing of internal clones helped determine the presence of polyadenylated transcripts at internal sites as well. The polyadenylation test and re-sequencing analysis showed that this phenomenon was not simply an artifact of the original data.

The poly (A) test, however, still uses oligo (dT) primers. Arguments can be made that the PCR in the poly (A) test is supported by internal priming of the oligo (dT) primers. However, PCR reactions using gene-specific primers designed flanking the poly (A) sites generated products with sizes exactly the same as predicted from the consensus sequence without internal poly (A/T) sequences, providing strong support evidence that the poly (A)-containing clones from the EST analysis were not derived from internal priming.

We have demonstrated that polyadenylation within the 28S rRNA of Ich occurs at three internal positions of the complete transcript as well as at the 3′ end of the transcripts. It is not clear why polyadenylation occurs at both internal and end of the transcripts. One possibility is that polyadenylation could serve dual functions as both a stability signal and degradation signal. For mRNA, it is widely believed that polyadenylation protects the mRNA from degradation in eukaryotes (Beelman and Parker, 1995; de Moor and Richter, 2001; Edmonds, 2002; Shatkin and Manley, 2000). However, with rRNA, polyadenylation could signal the degradation of the molecules, especially when they are present as truncated transcripts (Fleischmann et al., 2004; Kuai et al., 2004; LaCava et al., 2005; Slomovic et al., 2005; Slomovic et al., 2006; Vanacova et al., 2005; Wyers et al., 2005).

One obvious question is why polyadenylation occurs at the specific sites as observed. Are the processes sequence-dependent? Alignment of the sequence intervals between the observed polyadenylation positions failed to reveal any conserved sequences (Fig. 6) nor any conserved secondary structures, suggesting that it is sequence independent, as similarly reported in humans (Slomovic et al., 2006).

While the roles and mechanisms of polyadenylation in rRNA are unknown at present, polyadenylation of eukaryotic messages has a role in transcript stability, among other functions. Interestingly, there is evidence that the contrast is true of polyadenylation in some organellar RNA as well as prokaryotic and archeal messages; polyadenylation in these systems stimulates a decay or degradation cascade (Dreyfus and Regnier, 2002; Kushner, 2004; O'Hara et al., 1995; Slomovic et al., 2005). Additionally, polyadenylation could paradoxically serve as a signal for decay and to increase stability of a mature transcript in the same organism, specifically in humans and possibly in yeasts (Kao and Read, 2005; LaCava et al., 2005; Slomovic et al., 2005; Vanacova et al., 2005; West et al., 2006; Wyers et al., 2005). In Ich, there is perhaps evidence of a similar dual function for polyadenylation. The evidence supporting polyadenylation as a signal for degradation was the detection of truncated polyadenylated transcripts along various regions of the 28S rRNA, suggesting that many of these transcripts could be captured as intermediary degradation fragments. Since we assembled our contig using normalized sequences, we cannot assess the scope of polyadenlyated rRNAs *in vivo*. Further studies are needed to reveal the mechanisms and functions of polyadenylation in rRNAs of Ich.

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References

Abernathy, J.W., Xu, P., Li, P., Xu, D.H., Kucuktas, H., Klesius, P., Arias, C., Liu, Z., 2007. Generation and analysis of expressed sequence tags from the ciliate protozoan parasite *Ichthyophthirius multifiliis*. BMC Genomics 8, 176.

Beelman, C.A., Parker, R., 1995. Degradation of mRNA in eukaryotes. Cell 81, 179–183.

Decuypere, S., Vandesompele, J., Yardley, V., De Donckeri, S., Laurent, T., Rijal, S., Llanos-Cuentas, A., Chappuis, F., Arevalo, J., Dujardin, J.C., 2005. Differential polyadenylation of ribosomal RNA during post-transcriptional processing in *Leishmania*. Parasitology 131, 321–329.

de Moor, C.H., Richter, J.D., 2001. Translational control in vertebrate development. Int. Rev. Cytol. 203, 567–608.

Dreyfus, M., Regnier, P., 2002. The poly(A) tail of mRNAs: bodyguard in eukaryotes, scavenger in bacteria. Cell 111, 611–613.

Edmonds, M., 2002. A history of poly (A) sequences: from formation to factors to function. Prog. Nucleic Acid Res. Mol. Biol. 71, 285–389.

Eisen, J.A., Coyne, R.S., Wu, M., Wu, D., Thiagarajan, M., Wortman, J.R., Badger, J.H., Ren, Q., Amedeo, P., Jones, K.M., Tallon, L.J., Delcher, A.L., Salzberg, S.L., Silva, J.C., Haas, B.J., Majoros, W.H., Farzad, M., Carlton, J.M., Smith, R.K., Garg, J., Pearlman, R.E., Karrer, K.M., Sun, L., Manning, G., Elde, N.C., Turkewitz, A.P., Asai, D.J., Wilkes, D.E., Wang, Y., Cai, H., Collins, K., Stewart, B.A., Lee, S.R., Wilamowska, K., Weinberg, Z., Ruzzo, W.L., Wloga, D., Gaertig, J., Frankel, J., Tsao, C.C., Gorovsky, M.A., Keeling, P.J., Waller, R.F., Patron, N.J., Cherry, J.M., Stover, N.A., Krieger, C.J., Del Toro, C., Ryder, H.F., Williamson, S.C., Barbeau, R.A., Hamilton, E.P., Orias, E., 2006. Macronuclear genome sequence of the Ciliate *Tetrahymena thermophila*, a model eukaryote. PLoS Biol. 4 (9), e286.

Ewing, B., Green, P., 1998. Base-calling of automated sequencer traces using phred. II. Error probabilities. Genome Res. 8, 186–194.
Ewing, B., Hillier, L., Wendl, M.C., Green, P., 1998. Base-calling of automated sequencer traces using phred. I. Accuracy assessment. Genome Res. 8, 175–185.
Fleischmann, J., Liu, H., 2001. Polyadenylation of ribosomal RNA by *Candida albicans*. Gene 265, 71–76.
Fleischmann, J., Liu, H., Wu, C.P., 2004. Polyadenylation of ribosomal RNA by *Candida albicans* also involves the small subunit. BMC Mol. Biol. 5, 17.
Kao, C.Y., Read, L.K., 2005. Opposing effects of polyadenylation on the stability of edited and unedited mitochondrial RNAs in *Trypanosoma brucei*. Mol. Cell Biol. 25, 1634–1644.
Kuai, L., Fang, F., Butler, J.S., Sherman, F., 2004. Polyadenylation of rRNA in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. U.S.A. 101, 8581–8586.
Kushner, S.R., 2004. mRNA decay in prokaryotes and eukaryotes: different approaches to a similar problem. IUBMB Life 56, 585–594.
LaCava, J., Houseley, J., Saveanu, C., Petfalski, E., Thompson, E., Jacquier, A., Tollervey, D., 2005. RNA degradation by the exosome is promoted by a nuclear polyadenylation complex. Cell 121, 713–724.
Liu, Z.J., 2007a. Transcriptome characterization through the analysis of expressed sequence tags, In: Liu, Z.J. (Ed.), 1st ed. Aquaculture Genome Technologies, vol. 20. Blackwell Publishing, Ames, IA, pp. 339–354.
Liu, Z.J., 2007b. DNA sequencing technologies, In: Liu, Z.J. (Ed.), 1st ed. Aquaculture Genome Technologies, vol. 25. Blackwell Publishing, Ames, IA, pp. 463–474.
Mangus, D.A., Evans, M.C., Jacobson, A., 2003. Poly(A)-binding proteins: multifunctional scaffolds for the post-transcriptional control of gene expression. Genome Biol. 4, 223.
Matthews, R.A., 2005. *Ichthyophthirius multifiliis* Fouquet and ichthyophthiriosis in freshwater teleosts. Adv. Parasitol. 59, 159–241.
O'Hara, E.B., Chekanova, J.A., Ingle, C.A., Kushner, Z.R., Peters, E., Kushner, S.R., 1995. Polyadenylation helps regulate mRNA decay in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. 92, 1807–1811.
Prescott, D.M., 1994. The DNA of ciliated protozoa. Microbiol. Rev. 58, 233–267.
Salles, F.J., Richards, W.G., Strickland, S., 1999. Assaying the polyadenylation state of mRNAs. Methods 17, 38–45.
Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A laboratory Manual, 2nd Ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Shatkin, A.J., Manley, J.L., 2000. The ends of the affair: capping and polyadenylation. Nat. Struct. Biol. 7, 838–842.
Slomovic, S., Laufer, D., Geiger, D., Schuster, G., 2005. Polyadenylation and degradation of human mitochondrial RNA: the prokaryotic past leaves its mark. Mol. Cell Biol. 25, 6427–6435.
Slomovic, S., Laufer, D., Geiger, D., Schuster, G., 2006. Polyadenylation of ribosomal RNA in human cells. Nucleic Acids Res. 34, 2966–2975.
Vanacova, S., Wolf, J., Martin, G., Blank, D., Dettwiler, S., Friedlein, A., Langen, H., Keith, G., Keller, W., 2005. A new yeast poly (A) polymerase complex involved in RNA quality control. PLoS Biol. 3, e189.
West, S., Gromak, N., Norbury, C.J., Proudfoot, N.J., 2006. Adenylation and exosome-mediated degradation of cotranscriptionally cleaved pre-messenger RNA in human cells. Mol. Cell. 21, 437–443.
Wright, A.D., Lynn, D.H., 1995. Phylogeny of the fish parasite *Ichthyophthirius* and its relatives *Ophryoglena* and *Tetrahymena* (Ciliophora, Hymenostomatia) inferred from 18S ribosomal RNA sequences. Mol. Biol. Evol. 12, 285–290.
Wyers, F., Rougemaille, M., Badis, G., Rousselle, J.C., Dufour, M.E., Boulay, J., Regnault, B., Devaux, F., Namane, A., Seraphin, B., Libri, D., Jacquier, A., 2005. Cryptic pol II transcripts are degraded by a nuclear quality control pathway involving a new poly(A) polymerase. Cell 121, 725–737.
Xu, D.H., Klesius, P.H., 2004. Two year study on the infectivity of *Ichthyophthirius multifiliis* in channel catfish *Ictalurus punctatus*. Dis. Aquat. Org. 59, 131–134.